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(54) Amplifying and detecting target nucleic acids using a post amplification incubation step

(57) The present invention relates to a method for amplifying and detecting a target nucleic acid. The method comprising contacting a sample suspected of containing the target nucleic acid with a thermostable DNA polymerase and two primers that are substantially complementary to the target nucleic acid, under conditions such that the target nucleic acid is amplified. The amplified target nucleic acids are then denatured to form single stranded nucleic acids. Following amplification, the sample is subject to a pre-detection incubation step. The sample is incubated for between 1 second and 30 minutes at between 95°C and 120°C to inactivate said polymerization agent. Finally, the presence or absence of the amplified target nucleic acids is determined.

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Description

BACKGROUND INFORMATION

Field of the Invention

The present invention relates to a method for amplifying and detecting target nucleic acids. In particular, it relates to improved methods of detecting amplified nucleic acid products. The present invention can be used in various medical and research studies, forensic investigations, and diagnostic procedures, such as for the detection of genetic disorders or infectious diseases.

Background of the Invention

Technology to detect minute quantities of nucleic acids has advanced rapidly over the last two decades including the development of highly sophisticated hybridization assays using probes in amplification techniques such as polymerase chain reaction (PCR). Researchers have readily recognized the value of such technology to detect diseases and genetic features in human and animal test specimens. The use of primers and probes in the amplification and detection of nucleic acids is based upon the concept of complementarity, that is, the bonding of two strands of a nucleic acid by hydrogen bonds between complementary nucleotides (which are known as nucleotide pairs).

Much research has been carried out to find ways to amplify and detect small quantities of DNA. Various procedures are known and have been used to amplify or greatly multiply the number of nucleic acids in a specimen for detection. Such amplification techniques include PCR, ligase chain reaction (LCR), and branched DNA.

PCR is the most well known of these amplification methods. Details of PCR are well described in the art, including, for example, U.S. Patent Nos. 4,638,195 (Mullis et al.), 4,683,202 (Mullis), and 4,965,188 (Mullis et al.). Without going into extensive detail, PCR involves hybridizing primers to the strands of a target nucleic acid in the presence of polymerization agent (such as a DNA polymerase) and deoxyribonucleoside triphosphates under the appropriate conditions. The result is the generation of primer extension products along the templates, the products having added thereto nucleotides that are complementary to the templates.

Once the primer extension products are denatured and one copy of the templates has been prepared, the cycle of priming, extending and denaturation can be carried out as many times as desired to provide an exponential increase in the amount of nucleic acid that has the same sequence as the target nucleic acid. In effect, the target nucleic acid is duplicated (or "amplified") many times so that it is more easily detected. Once the target nucleic acid has been sufficiently amplified, various detection procedures can be used to detect, qualitatively and/or quantitatively, the presence of the target.

Once the target nucleic acid has been sufficiently amplified, various detection procedures can be used to detect its presence. A standard detection method used to detect PCR products has been ethidium bromide stained agarose gels. Use of ethidium bromide stained gels, however, has several disadvantages including, for example, relatively poor sensitivity and specificity.

Improved methods of detecting PCR products that eliminate the use of radiolabels and electrophoresis have been developed. These nonisotopic oligonucleotide capture detection methods rely on specific hybridization to probes and enzymatic signal generation. Such nonisotopic oligonucleotide capture detection methods, also known as reverse dot blot detection, are described in U.S. Patent Nos. 5,229,297 (Schnepilsky et al.), 5,328,825 (Warren et al.), and 5,422,271 (Chen et al.). Such a method is also described in Findlay et al., Clinical Chemistry, 39:1927-1933 (1993).

These nonisotopic detection methods have higher sensitivity and specificity than ethidium bromide staining detection and avoid the use of radioactivity. The methods operate by either carrying out amplification with biotinylated primer (s) or using a biotinylated probe to detect the amplified nucleic acids. Biotinylated products or probes are subsequently reacted with an avidin or streptavidin conjugated enzyme such as horseradish peroxidase (HRP). A dye precursor (or light generating signal reagent) can then be brought into contact with the enzyme and be converted into a dye (luminescence) thereby generating a detectable signal.

Nonisotopic oligonucleotide capture detection methods are not, however, without their own drawbacks. If nonisotopic oligonucleotide capture detection is carried out utilizing standard PCR denaturation conditions (95°C) to denature concentrated or minimally diluted amplified nucleic acid products, the enzymes utilized to carry out the amplification reaction, such as thermostable polymerases or DNA ligases, will still be present and active. The presence of such active enzymes during detection results in binding competition between the enzyme and the probe for the amplification product. Such competition can reduce the amount of amplified nucleic acid products bound to probe and therefor, the detection signal.

One solution to this problem has been to add high levels of ethylenediamine tetraacetic acid (EDTA), a chelator of Mg^{++}, to the PCR amplification mixture after amplification has been carried out but prior to detection. EDTA is able

to inhibit many enzymes requiring Mg^{++} for activity including DNA polymerases and DNA ligases. Use of EDTA, however, adds an additional step to the PCR amplification and detection process. In addition, use of EDTA requires opening up the reaction vessel to add the EDTA. As those skilled in the art are aware, opening the reaction vessel is to be avoided because of contamination concerns.

Thus, blocking the amplification process during detection through the addition of EDTA or other such enzyme inhibitors is not desired. Rather, it is desirable to have a method of inactivating the amplification enzymes prior to detection without the increased risk of contamination.

SUMMARY OF THE INVENTION

It is an object of the present invention to overcome the problems noted above by using a post amplification incubation step prior to detection to inactivate the amplification enzymes.

In one embodiment, the present invention relates to a method for amplifying and detecting a target nucleic acid comprising:

(i) contacting a sample suspected of containing the target nucleic acid with at least two oligonucleotides and a thermostable amplification enzyme, wherein the oligonucleotides are substantially complementary to a portion of the target nucleic acid, under conditions such that the target nucleic acid is amplified;
(ii) denaturing amplified target nucleic acids to form single stranded nucleic acids;
(iii) incubating the sample for between 1 second and 30 minutes at between 95°C and 120°C, as a post amplification incubation step to inactivate the thermostable amplification enzyme; and
(iv) detecting the presence or absence of the amplified target nucleic acids.

In a further embodiment, the present invention relates to a method for amplifying and detecting a target nucleic acid comprising:

(i) contacting a sample suspected of containing the target nucleic acid with four different nucleoside triphosphates, a thermostable DNA polymerase, and at least two primers, wherein the primers are substantially complementary to the target nucleic acid, under conditions such that the target nucleic acid is amplified;
(ii) denaturing amplified target nucleic acids to form single stranded nucleic acids;
(iii) incubating the sample for between 1 second and 30 minutes at between 95°C and 120°C, as a post amplification incubation step to inactivate the polymerization agent; and
(iv) detecting the presence or absence of the amplified target nucleic acids.

In another embodiment, the present invention relates to a method for amplifying and detecting a target nucleic acid comprising:

(i) contacting a sample suspected of containing target nucleic acid with four different nucleoside triphosphates, a thermostable DNA polymerase, and at least two primers, wherein at least one of the primers is labeled with biotin and all primers are substantially complementary to the target nucleic acid, under conditions such that the target nucleic acid is amplified;
(ii) incubating the sample for between 0.5 minutes and 5 minutes at about 105°C, as a post amplification incubation step to inactivate the polymerase; and
(iii) detecting the presence or absence of the biotinylated amplified target nucleic acids by reacting the biotinylated amplified target nucleic acids with a streptavidin-enzyme conjugate, followed by reaction of the enzyme with a substrate reagent to produce a detectable colorimetric or chemilluminescent signal.

Various other objects and advantages of the present invention will be apparent from the following description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The general principles and condition for amplification and detection of nucleic acids using polymerase chain reaction are quite well known, the details of which are provided in numerous references including U.S. Patent Nos 4,683,195 (Mullis et al.), 4,683,202 (Mullis), and 4,965,188 (Mullis et al.), all of which are incorporated herein by reference. Thus, in view of the teaching in the art and the specific teaching provided herein, a worker skilled in the art should have no difficulty in practicing the present invention by adding a post amplification incubation step to inactivate the amplification enzymes prior to product detection as taught herein to increase detection sensitivity.

Other amplification and detection procedures employing thermostable enzymes can also be used in the practice of this. invention. The present invention provides for a post amplification, pre-detection incubation step that inactivates the thermostable enzyme(s) used during amplification. Thus, the present invention is suitable for use with any amplification method employing a thermostable enzyme. Other thermostable amplification methods include ligase chain reaction (LCR) as described, for example, in EP-A-0 320 308 (published December, 1987) and EP-A-0 439 182 (published January, 1990), which uses a thermostable DNA ligase to ligate adjoining probes thereby creating a complementary nucleic acid sequence. In LCR target nucleic acids are amplified using 4 oligonucleotide probes and a thermostable DNA ligase. Two of the oligonucleotide probes are complementary to adjacent sites on one strand of the DNA template to be amplified. These probes hybridize to that DNA strand such that a nick is formed between the two probes. The nick is then sealed by a thermostable DNA ligase thereby creating a new strand of DNA complementary to the target. The third and fourth probes are complementary to the second strand of the DNA template and function like the first pair of probes to generate a complementary DNA. The amplified products of LCR can be detected using standard detection methods. The post amplification, pre-detection incubation step of the present invention can be used with LCR to inactivate the DNA ligase prior to detection. Thus, the teachings provided herein would allow one skilled in the art to adapt the post amplification enzyme denaturation step shown for PCR to these other known amplification and detection procedures. The remainder of this disclosure is directed to practicing the invention using PCR for illustrative purposes.

The present invention provides a modification of known methods of PCR in order to improve detection sensitivity. It has been surprisingly discovered in accordance with the present invention that a post amplification, pre-detection incubation step can be used to inactivate the polymerization agent and reduce the binding competition between the probe and the agent for the amplified target nucleic acids. This reduced competition increases detection sensitivity.

The present invention is directed towards the amplification and detection of one or more target nucleic acids present in a test specimen. Test specimens can include cellular or viral material, body fluids or other cellular materials containing genetic DNA or RNA that can be detected.

Nucleic acids to be amplified and detected can be obtained from various sources including plasmids and naturally occurring DNA or RNA from any source (such as bacteria, yeast, viruses, plants, higher animals, or humans). It may be extracted from various tissues including but not limited to, blood, peripheral blood mononuclear cells (PBMC), tissue material or other sources known in the art using known procedures. The present invention is particularly useful for the amplification and detection of one or more nucleic acid sequences found in genomic DNA, bacterial DNA, fungal DNA, viral RNA, or DNA or RNA found in bacterial or viral infected cells.

The method described herein can be used to amplify and detect target nucleic acids associated with infectious diseases, genetic disorders, and cellular disorders such as cancer. It may also be used for forensic investigations and DNA typing. It is particularly useful for the detection of infectious agents, such as bacteria and viruses, by detection of nucleic acids associated therewith. It has particular utility when very high sensitivity and/or quantitation is required.

Bacteria that can be detected by the present invention include, but are not limited to, bacteria found in human blood, such as Salmonella species, Streptococcus species, Chlamydia species, Gonococcal species, Mycobacteria species (such as, *Mycobacterium tuberculosis* and *Mycobacterium avium* complex), Mycoplasma species (such as *Mycoplasma Hemophilus influenzae* and *Mycoplasma pneumoniae), Legionella pneumophila, Borrelia burgdorferei, Pneumocystis carinii, Clostridium difficile,* Camplyobacteri species, Yersinia species, Shigella species and Listeria species. Viruses that are detectable include, but are not limited to, cytomegalovirus, herpes simplex virus, Epstein Barr virus, human papilloma viruses, influenza viruses, hepatitis viruses, and retroviruses (such as, HTLV-I, HTLV-II, HIV-I and HIV-II). Protozoan parasites, yeasts and molds are also detectable by the present invention. Other detectable species would be readily apparent to one skilled in the art.

A "PCR reagent" refers to any of the reagents considered essential for PCR, namely a set of primers for each target nucleic acid, a DNA polymerase, a DNA polymerase cofactor, and one or more deoxyribonucleoside-5'-triphosphates (dNTP's). Other optional reagents and materials used in PCR are described below.

The term "primer" refers to an oligonucleotide, whether naturally occurring or synthetically produced, that is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand (that is, template) is induced, such conditions include the presence of other PCR reagents, and suitable temperature and pH.

The primers of the present invention are selected to be "substantially complementary" to the specific nucleic acid sequence to be primed and amplified. This means that they must be sufficiently complementary to hybridize with the respective nucleic acid sequences to form the desired hybridized products and then be extendable by a DNA polymerase. Typically, a "substantially complementary" primer will contain at least 70% or more bases which are complementary to the target sequence. More preferably 80% of the bases are complementary, and even mcre preferably 90% of the bases are complementary. In the most preferred situations, the primers have between 90% and 100% exact complementarity to the target nucleic acid sequence.

The primer is preferably single stranded for maximum efficiency in amplification, but can contain a double stranded

region if desired. It must be long enough to prime the synthesis of extension products in the presence of the DNA polymerase. The exact size of each primer will vary depending upon the use contemplated, the concentration and sequence of the primer, the complexity of the targeted sequence, the reaction temperature, and the source of the primer. Generally, the primers used in this invention will have from 12 to 60 nucleotides, and preferably, they have from 16 to 40 nucleotides. More preferably, each primer has from 18 to 35 nucleotides.

Primers useful herein can be prepared using known techniques and equipment, including for example an ABI DNA Synthesizer (available from Applied Biosystems) or a Biosearch 8600 Series or 8800 Series Synthesizer (available from Milligen-Biosearch, Inc.). Procedures for using this equipment are well known and described for example in U.S. Patent No. 4,965,188 (Gelfand et al.), incorporated herein by reference. Naturally occurring primers isolated from biological sources may also be useful (such as restriction endonuclease digests). A set of at least two primers is generally used for each target nucleic acid. Thus, a plurality of sets of primers can be used simultaneously to amplify a plurality of target nucleic acids.

As used herein, a "probe" is an oligonucleotide which is substantially complementary to a nucleic acid sequence of the target nucleic acid and which is used for detection or capture of the amplified target nucleic acid.

The primers and/or the probes used in the present invention can, optionally, be labeled. Using known methods in the art, the primers and/or probes can be labeled with a specific binding ligand (such as biotin), an enzyme (such as glucose oxidase, peroxidases, uricase, and alkaline phosphatase), radioisotopes, electron-dense reagents, chromogens, fluorogens, phosphorescent moieties or ferritin. Preferably, the label is a specific binding ligand. More preferably, the label is biotin or a derivative thereof, streptavidin or a derivative thereof or a hapten.

Additional PCR reagents necessary for PCR include a DNA polymerase (preferably a thermostable DNA polymerase), a DNA polymerase cofactor and appropriate dNTP's. These reagents can be provided individually, as part of a test kit, or in reagent chambers of test devices.

A DNA polymerase is an enzyme that will add deoxynucleoside monophosphate molecules to the 3'- hydroxy end of the primer in a complex of primer and template, but this addition is in a template dependent manner. Generally, synthesis of extension products proceeds in the 5' to 3' direction of the newly synthesized strand until synthesis is terminated. Useful DNA polymerases include, for example, *E. coli* DNA polymerase I, T4 DNA polymerase, Klenow polymerase, reverse transcriptase and others known in the art. Preferably, the DNA polymerase is thermostable meaning that it is stable to heat and preferentially active at higher temperatures, especially the high temperatures used for priming and extension of DNA strands. More particularly, thermostable DNA polymerases are not substantially inactive at the high temperatures used in polymerase chain reactions as described herein. Such temperatures will vary depending on a number of reaction conditions, including pH, nucleotide composition, length of primers, salt concentration and other conditions known in the art.

A number of thermostable DNA polymerases have been reported in the art, including those mentioned in detail in U.S. Patent Nos. 4,965,188 (Gelfand et al.) and 4,889,818 (Gelfand et al.), both incorporated herein by reference. Particularly useful polymerases are those obtained from various Thermus bacterial species, such as *Thermus aquaticus, Thermus thermophilus, Thermus filiformis,* and *Thermus flavus.* Other useful thermostable polymerases are obtained from various microbial sources including *Thermococcus literalis*, *Pyrococcus furiosus,* Thermotoga sp. and those described in WO-A-89/06691 (published July 27, 1989). Some useful thermostable polymerases are commercially available, such as, AppliTaq®, Tth, and UITma™ from Perkin Elmer, Pfu from Stratagene, and Vent and Deep-Vent from New England Biolabs. A number of techniques are also known for isolating naturally-occurring polymerases from organisms, and for producing genetically engineered enzymes using recombinant techniques.

A DNA polymerase cofactor refers to a nonprotein compound on which the enzyme depends for activity. Thus, the enzyme is catalytically inactive without the presence of cofactor. A number of materials are known cofactors including, but not limited to, manganese and magnesium salts, such as chlorides, sulfates, acetates and fatty acids salts. Magnesium chlorides and sulfates are preferred.

Also needed for PCR are two or more deoxyribonucleoside-5'-triphosphates, such as two or more of dATP, dCTP, dGTP, dTTP and dUTP. Analogues such as dITP and 7-deaza-dGTP are also useful. Preferably, the four common triphosphates (dATP, dCTP, dGTP and dTTP) are used together.

The PCR reagents described herein are provided and used in PCR in suitable concentrations to provide amplification of the target nucleic acid. The minimal amounts of primers, DNA polymerase, cofactors and deoxyribonucleoside-5'-triphosphates needed for amplification and suitable ranges of each are well known in the art. The minimal amount of DNA polymerase is generally at least about 0.5 units/100 µl of solution, with from about 2 to about 25 units/100 µl of solution being preferred, and from about 7 to about 20 units/100 µl of solution being more preferred. Other amounts may be useful for given amplification systems. A "unit" is defined herein as the amount of enzyme activity required to incorporate 10 nmoles of total nucleotides (dNTP's) into an extending nucleic acid chain in 30 minutes at 74°C. The minimal amount of primer is at least about 0.075 µmolar with from about 0.1 to about 2 µmolar being preferred, but other amounts are well known in the art. The cofactor is generally present in an amount of from about 2 to about 15 mmolar. The amount of each dNTP is generally from about 0.25 to about 3.5 mmolar.

The PCR reagents can be supplied individually, or in various combinations, or all in a buffered solution having a pH in the range of from about 7 to about 9, using any suitable buffer, many of which are known in the art.

Other reagents that can be used in PCR include, for example, antibodies specific for the thermostable DNA polymerase, exonucleases and/or glycosylases. Antibodies can be used to inhibit the polymerase prior to amplification. Antibodies useful in the present invention are specific for the thermostable DNA polymerase, inhibit the enzymatic activity of the DNA polymerase at temperatures below about 50°C, and are deactivated at higher temperatures. Useful antibodies include, monoclonal antibodies, polyclonal antibodies and antibody fragments. Preferably, the antibody is monoclonal. The antibodies useful in the present invention can be prepared using known methods such as those described in Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor, NY (1988).

Representative monoclonal antibodies are described in U.S. Patent No. 5,338,671 (Scalice et al.), the contents of which are hereby incorporated by reference. Two such monoclonal antibodies are readily obtained by a skilled artisan using conventional procedures, and starting materials including either of hybridoma cell lines HB 11126 or 11127, deposited with the American Type Culture Collection (ATCC) (Rockville, MD). The monoclonal antibody is present in an amount of from about 5:1 to about 500:1 molar ratio to the DNA polymerase.

Antibodies specific to the thermostable DNA polymerase can be used in the present invention alone or in combination with an exonuclease and/or a glycosylase as described in EP-A-0 726 324.

The combined use of an antibody, an exonuclease and a glycosylase reduces the formation of zero cycle artifacts. Suitable exonucleases for use in PCR include, but are not limited to, exonuclease III, exonuclease I, exonuclease, T7 exonuclease, ribonuclease II, polynucleotide phosphorylase and BAL 31 nuclease. Such exonucleases are commercially available or can be obtained by methods known in the art. Glycosylases useful in the present invention are those that specifically cleave unconventional bases, i.e., bases other than A, G, C, or T in DNA and A, G, C, and U in RNA. Preferred glycosylases include uracil-N-glycosylase (UNG), hypoxanthine-DNA glycosylase and 3-methyadenine-DNA glycosylases I and II. In a preferred embodiment, Taq polymerase, a monoclonal antibody against Taq polymerase, exonuclease III and uracil-N-glycosylase are employed.

A target nucleic acid (either DNA or RNA) can be obtained from any of a variety of sources as noted above. Generally, the sample is treated in some manner to make the DNA available for contact with the primers and other PCR reagents. This usually means removing unwanted proteins and cellular matter from the sample using one of the various procedures known in the art.

Since the nucleic acid to be amplified and detected is often in double stranded form, the two strands must be separated (that is, denatured) before priming and amplification can take place. Denaturation can be accomplished using a heat treatment alone or in combination with any other suitable physical, chemical or enzymatic means for separating the strands as described in the art. Initial denaturation is generally carried out by heating the sample suspected of containing the target nucleic acid at a first temperature of from about 85° to about 100°C for a suitable time, for example, from about 1 second to 3 minutes.

The denatured strands are then cooled to a temperature which-is generally in the range of from about 55° to about 70°C for priming of the strands. The time needed for cooling the strands after the initial denaturation will vary depending upon the type of apparatus used for the PCR process.

Once the denatured strands are cooled to the second temperature, the denatured strands are incubated together with the reaction mixture containing PCR reagents at a suitable temperature to effect annealing (hybridization) of the primers to the strands and extension of the primers to form primer extension products. Generally, this temperature is at least about 50°C, and preferably in the range of from about 62° to about 75°C. The time for incubation can vary widely depending upon the incubation temperature and the length of extension products desired, but in preferred embodiments, it is from about 1 to about 120 seconds. Each cycle of PCR can be carried out using either two or three different temperatures, one for denaturation, and a second or third temperature for priming and/or primer extension product formation.

At any point after the generation of at least one primer extension product, amplification can be stopped and the target primer extension product (the "amplified" target) detected. However, if the hybridized primer extension products are then denatured, PCR can be carried out further in as many cycles of priming, extending, and denaturing as desired. The number of PCR cycles carried out will depend, in part, upon the amount of amplified target desired and can be readily determined by those skilled in the art. Generally, at least 20 cycles will be carried cut, with from 20 to 50 cycles being preferred.

When amplifying multiple target nucleic acids, especially instances where one of the targets is a lower copy number target and one is a high copy number target, a secondary renaturation step can be employed after primary PCR cycles have been carried out, as described in EP-A-0 694 617.

After at least 15 primary amplification cycles (a primary amplification cycle comprising denaturation, priming and extension), secondary amplification cycles are carried out having the same steps, except that a renaturation step is included after each denaturation step and before primer annealing. Renaturation is accomplished by cooling the reaction mixture to a fourth temperature as described in EP-A-0 694 617.

In the present invention, after the target nucleic acid is amplified using the desired number of PCR cycles, a post amplification, pre-detection incubation step is performed to inactivate the DNA polymerase and, thereby, increase detection sensitivity. The conditions under which the post amplification incubation step is carried out will depend upon the thermostable enzyme employed but the combined temperature and incubation period will be such as to inactivate the enzyme. Preferably, this post amplification incubation step is carried out by incubating the PCR amplification mixture containing the amplified target at a temperature of between about 95°C and about 120°C for between about 1 second and about 30 minutes. Preferably, the post amplification incubation step involves heating at a temperature of between 100°C and 110°C for 15 seconds to 10 minutes, more preferably at a temperature of about 105°C for up to 5 minutes.

Once the post amplification incubation has been performed, the amplified nucleic acid targets can be detected. Detection can be accomplished in a number of known ways, such as those described in U.S. Patent No. 4,965,188 (Gelfand et al.). For example, the amplified nucleic acids can be detected using Southern blotting, dot blot techniques, or nonisotopic oligonucleotide capture detection with a labeled probe. Alternatively, amplification can be carried out using primers that are appropriately labeled, and the amplified primer extension products can be detected using procedures and equipment for detection of the label.

In a preferred embodiment, the amplified target nucleic acid is detected using an oligonucleotide probe that is labeled for detection and can be directly or indirectly hybridized with the amplified target. The probe may be soluble or attached to a solid support. In another preferred embodiment, one or more of the primers used to amplify the target nucleic acid is labeled, for example, with a specific binding moiety. The resulting primer extension product into which the labeled primer has been incorporated can be captured with a probe. Detection of the amplified target hybridized to the probe can be achieved by detecting the presence of the labeled probe or labeled amplified target using suitable detection equipment and procedures that are well known in the art. Certain labels may be visible to the eye without the use of detection equipment.

In a more preferred embodiment, one or more of the primers used to amplify the target nucleic acid is labeled with biotin and the biotinylated amplified target nucleic acids are hybridized to probes attached to a solid support. The bound targets are then detected by contacting them with a streptavidin-peroxidase conjugate in the presence of an oxidant, such as hydrogen peroxide, and a suitable dye-forming composition. For example, useful dye-providing reagents include tetramethylbenzidine and derivatives thereof, and leuco dyes, such as triarylimidazole leuco dyes as described in U.S. Patent No. 4,089,747 (Bruschi).

As used herein, when in reference to time the term "about" refers to ± 10% of that time limit. When used in reference to temperatures, the term "about" refers to ± 5°C.

The following examples are included to illustrate the practice of this invention, and are not meant to be limiting in any way. All percentages are by weight unless otherwise indicated.

EXAMPLES

Materials

Recombinant DNA polymerase from *Thermus aquaticus* was prepared using, known procedures, such as that described in EP-A-0 482 714, and had an activity of about 250,000 units/mg of protein.

The primers used in the following Examples had the following sequences:

5'-CACCACGCAGCGGCCCTTGATGTTT-3' (SEQ. ID NO. 1),

5'-TGCACTGCCAGGTGCTTCGGCTCAT-3' (SEQ. ID NO. 2.)

The capture probe has the following sequence:

5'-GAACCGAGGGCCGGCTCACCTCTATGTTGG-3' (SEQ. ID NO. 3).

The primers and probes used in the following Examples were prepared using known starting materials and procedures using an Applied Biosystems Model 380B, three column DNA synthesizer using standard phosphoramidite chemistry and the ABI 1μ molar scale, fast cycle protocol. Nucleoside-3'-phosphoramidites and nucleoside derivatized controlled pore glass supports were obtained from Applied Biosystems. The primers had the sequences identified above. They were functionalized at the 5' end with two amino tetraethylene glycol spacers according to U.S. Patent 4,962,029, followed by a single commercially available DuPont biotin phosphoramidite. The probes were functionalized

at the 3'end with two tetraethylene glycol spacers followed by a single aminodiol linking group according to U.S. Patent No. 4,914,210. All purifications were carried out using a nucleic acid purification column, followed by reverse phase HPLC techniques. Deoxyribonucleotides (dNTP's) were obtained from Sigma Chemical Co.

A streptavidin-peroxidase conjugate solution was used that comprised a commercially available (Sigma Chemical Co.) conjugate of steptavidin and horseradish peroxidase, casein (0.5%), and merthiolate (0.5%) in a phosphate buffered saline solution (24 mmolar sodium phosphate and 75 mmolar sodium chloride). 10 mmolar 4'-hydroxyacetanilide was added as a conjugate stabilizer. In Examples 1 and 2 the final conjugate concentration was 1.1 nM. In Example 3 the final conjugate concentration was 0.28 nM.

Cytomegalovirus, strain AD 169 DNA was received from Applied Biotechnology Inc. Briefly, the DNA was extracted from human foreskin fibroblast cell lines using conventional procedures:

Virus Lot Specifications

Virus:	Cytomegalovirus, strain AD 169
Cell Line for Propagation:	Human Foreskin Fibroblasts
Virus Preparation:	Sucrose density gradient purified, 1000x concentration
Virus Particle Count:	1.65×10^{10} vp/mL at 1000x
$TCID_{50}$ Titre on Active Virus:	10^7 $TCID_{50}$ units/mL at 1000x

DNA Extract Specifications

Volume:	0.1 mL
Suspending Buffer:	10 mM Tris/ 1mM EDTA, pH 8.0
Extract Preparation:	SDS, proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. One mL of extract prepared from 1 mL of purified virus.
Shipping and Storage:	6 x 0.1 mL shipped frozen at -70°C. Stored at -20°C or colder.

The leuco dye dispersion contained agarose (0.5%), 4,5-bis(4-dimethylaminophenyl)-2-(4 hydroxy-3-methoxyphenyl)imidazole leucodye (250 µmolar), diethylenetriamine pentaacetic acid (100 µmolar), 3'-chloro-4'-hydroxyacetanilide (5 mmolar), polyvinylpyrrolidone (112 mmolar), and sodium phosphate, monobasic, 1-hydrate (10 mmolar) and hydrogen peroxide (H_2O_2) (8.82 mmolar).

The wash solution (pH 7.4) contained sodium chloride (373 mmolar), (ethylenedinitrilo)tetraacetic acid disodium salt (2.5 mmolar), decyl sodium sulfate (38 mmolar) and ethylcerithio salicylic acid, sodium salt (25 µmolar)in sodium phosphate, monobasic 1-hydrate buffer (25 mmolar).

Monoclonal antibodies were used in the reaction mixture. These antibodies "TP1-12.2" and TP4-9.2" are specific to DNA polymerase from *Therms acquaticus* and are described in more detail in EP-A-0 726 324.

The polymerase chain reaction mixture (75 mL) contained tris(hydroxymethyl)aminomethane buffer (10 mmolar, pH 8), potassium chloride (50 mmolar), magnesium chloride (4 mmolar), dATP, dCTP, dGTP, and dTTP (0.3 mM each), the primers SEQ ID NO: 1 and SEQ ID NO:2 (0.4 µmolar each), Type IV gelatin (100 mg/mL), Taq polymerase (16 units/100 µl), and glycerol (9.5%). A fifty fold molar excess (over polymerase) of TP1-12.2 and a 5X excess of TP4-9.2 were used.

PCR amplification was carried out using an automated PCR processor described in U.S. Patent No. 5,089,233.

To form capture reagents, the probes were covalently attached to polymeric particles (1 µm average diameter) prepared using conventional emulsion polymerization techniques, from poly[styrene-co-3(p-vinyl-benzylthio)propionic acid] (95:5 weight ratio, 1µm average diameter). A suspension of the particles in water was washed with 2-(N-morpholino)ethanesulfonic acid buffer (0.1 molar, pH6), and suspended to about 10% solids. A sample (3.3 ml) of the washed particles, diluted to 3.33% solids in the buffer (0.1 molar, was mixed with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.1 ml of 84 mg/ml water) and the probe 983 µl of 44.44 OD/ml nanopure water). The resulting suspension was heated at 50°C in a water bath for about two hours with intermittent mixing and centrifuged. The particles were then washed three times with tris(hydroxymethyl)aminomethane buffer (0.01 molar, pH 8) containing (ethylenedinitrilo)tetraacetic acid disodium salt (0.001 molar) and resuspended therein to 4% solids. The particles were then immobilized in discrete spots in Clinical Diagnostic's PCR pouch at 2% solids plus glue. The PCR products were detected using the Clinical Diagnostic's Pouch detection system.

Other reagents and materials were cbtained either from commercial sources or prepared using readily available starting materials and conventional procedures.

Post Amplification Incubation Step Prior to Product Detection for Increased Detection Sensitivity

Example 1

This example demonstrates the present invention to detect nucleic acid products that have been produced using PCR by employing a post amplification incubation step to denature the polymerase.

Positive pools were created by amplifying CMV target using 40-45 cycles of the following PCR protocol:

1. Denaturation by heating at 95°C for 15 seconds, and
2. Cycles of priming and extending at 70°C for 30 seconds.

Product concentration was quantified by gel electrophoresis with known concentrations of DNA standards. The resulting post amplification PCR reaction mixture was then used as described below.

In addition to generating the post-PCR reaction mixture, a CMV negative product pool was prepared by carrying out PCR amplification on the PCR reaction mixture absent the addition of CMV DNA target using the above PCR protocol.

The post amplification PCR reaction mixture (10^{-7} to 10^{-8}M) was diluted 1:100 to 1:5000 with CMV negative product pool to obtain a final CMV DNA concentration of 1×10^{-10} M, 2.5×10^{-11} M, or 5×10^{-11} M. The diluted post amplification PCR reaction mixture was then subjected to a post amplification incubation to deactivate the polymerase. The post-amplification incubation step was carried out for 2 minutes at 97°C or 100°C, or for 5 minutes at 100°C.

After the post amplification incubation, the amplified product was detected by capturing the target nucleic acids with the capture reagents at 58°C for 5 minutes inside a Clinical Diagnostic's PCR pouch. The captured products were then contacted and incubated with the streptavidin-peroxidase conjugate solution at 55°C for 1 minute. A wash was carried out using the wash solution for 1 minute at 55°C, after which the dye-providing composition was added and allowed to incubate for 4 minutes at 40°C. The resulting signal was read with a line array scanner. The scanner determined the change in reflectance density (ΔDr). ΔDr is the difference in the reflectance density between an initial reading before initiation of dye development and a final reading taken after 4 minutes of dye development. The scanner background on visually negative capture beads ranged from 0.05 to 0.1 Dr units.

The following results show that a post amplification incubation step increases detection sensitivity:

Post amplification Incubation Condition	Product Concentration	CMV _Dr (Scanner)
2 min at 97°C	1×10^{-10} M	0.175
"	5×10^{-11} M	0.14
"	2.5×10^{-11} M	0.11
2 min at 100°C	1×10^{-10} M	0.265
"	5×10^{-11} M	0.19
"	2.5×10^{-11} M	0.13
5 min at 100°C	1×10^{-10} M	0.435
"	5×10^{-11} M	0.31
"	2.5×10^{-11} M	0.21

These results show that a 5 minute post amplification incubation step at 100°C increases the effective detection limit above background by at least four-fold and probably by at least five-fold. Based on these results, especially the improvement by increasing the incubation period at 100°C from 2 minutes to 5 minutes, a second experiment was carried out with a 15 minute post amplification incubation at 100°C.

Example 2

In this second experiment, amplification of CMV DNA was carried out as described in Example 1. The resulting post amplification PCR reaction mixture was diluted with a CMV negative product pool and subjected to a post amplification incubation to inactivate the polymerase as described in Example 1. The post-amplification incubation step was carried out for 15 minutes at 100°C. After the post amplification incubation, the amplified product was detected as described in Example 1.

The following results show that a post amplification incubation step increases detection sensitivity:

Post amplification Incubation Condition	Product Concentration	CMV _Dr (Scanner)
2 min at 97°C	1 x 10^{-10} M	0.140
"	1 x 10^{-10} M	0.122
5 min at 100°C	1 x 10^{-10} M	0.336
"	1 x 10^{-10} M	0.346
15 min at 100°C	1 x 10^{-10} M	0.503
"	1 x 10^{-10} M	0.480

These results suggest that increasing the post amplification incubation time at 100°C gains additional benefits.

Example 3

To determine whether an additional detection sensitivity benefit could be realized by increasing the incubation temperature, amplification of CMV DNA was carried out as described in Example 1 and subjected post amplification incubation at 100°C or 103°C. Various incubation times at 103°C were investigated. In addition, this experiment was carried out with a less sensitive detection chemistry that has a final conjugate concentration of 0.28 nM. The results of this experiment were:

Post Amplification Incubation Condition	Product Concentration	CMV _Dr (Scanner)
5 min at 100°C	1 x 10^{-10} M	0.152
15 min at 100°C	1 x 10^{-10} M	0.270
2 min at 103°C	1 x 10^{-10} M	0.264
5 min at 103°C	1 x 10^{-10} M	0.318

These results demonstrate that at least an additional two-fold increase, and probably a three-fold increase, can be attained by increasing the post amplification incubation step temperature from 100°C to 103°C and maintaining the five minute incubation period.

All publications mentioned hereinabove are hereby incorporated by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by those skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Johnson & Johnson Clinical Diagnostics, Inc.
(B) STREET: 100 Indigo Creek Drive
(C) CITY: Rochester
(D) STATE: New York
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): NY 14650

(ii) TITLE OF INVENTION: Amplifying and detecting target nucleic acids using a post amplification incubation step

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk 720K
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 97301591.0

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CACCACGCAG CGGCCCTTGA TGTTT 25

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGCACTGCCA GGTGCTTCGG CTCAT 25

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAACCGAGGG CCGGCTCACC TCTATGTTGG 30

Claims

1. A method for amplifying and detecting a target nucleic acid comprising:

(i) either (a) contacting a sample suspected of containing said target nucleic acid with at least two oligonucleotides and a thermostable amplification enzyme, wherein said at least two oligonucleotides are substantially complementary to a portion of said target nucleic acid, under conditions such that said target nucleic acid is amplified

or (b) contacting a sample suspected of containing said target nucleic acid with four different nucleoside triphosphates, a thermostable DNA polymerase, and two primers, wherein said primers are substantially complementary to said target nucleic acid, under conditions such that said target nucleic acid is amplified;
(ii) denaturing amplified target nucleic acids to form single stranded nucleic acids;
(iii) incubating said sample for between 1 second and 30 minutes at between 95°C and 120°C, as a post amplification incubation step to inactivate said thermostable amplification enzyme; and
(iv) detecting the presence or absence of said amplified target nucleic acids.

2. The method of claim 1, wherein four oligonucleotides and a thermostable DNA ligase are used.

3. The method of claim 1 or claim 2, wherein said post amplification incubation step is carried out for between 15 seconds to 10 minutes, preferably for between 0.5 minutes to 5 minutes, at between 100°C to 110°C, preferably at about 105°C.

4. The method of any one of claims 1 to 3, wherein said target nucleic acid is DNA or RNA.

5. The method of any one of claims 1 to 4, wherein said nucleoside triphosphates are deoxyribonucleoside triphosphates, and preferably are dATP, dCTP, dGTP and dTTP.

6. The method of any one of claims 1 to 5, wherein said thermostable DNA polymerase is selected from the group consisting of *thermus aquaticus* polymerase, thermus *thermophilus* polymerase, and *thermococcus litoralis* polymerase.

7. The method of any one of claims 1 to 6, wherein at least one and preferably both of said primers is labeled.

8. The method of claim 7, wherein at least one of said primers is labeled with a specific binding ligand, which preferably is biotin.

9. The method of any one of claims 1 to 8, wherein said amplified target nucleic acids are detected using a labeled probe that can hydrodize with one of the one or more target nucleic acids.

10. The method of any one of claims 1 to 8, wherein at least one of said primers is labeled with a specific binding moiety and said amplified target nucleic acids are detected using a probe that can hybridize with one of the one or more target nucleic acids.

11. The method of claim 9 or claim 10, wherein said probe is attached to a solid support.

12. A method for amplifying and detecting a target nucleic acid comprising:

(i) contacting a sample suspected of containing target nucleic acid with four different nucleoside triphosphates, a thermostable DNA polymerase, and two primers, wherein at least one of said primers is labeled with biotin and said primers are substantially complementary to said target nucleic acid, under conditions such that said target nucleic acid is amplified;
(ii) incubating said sample for between 0.5 minutes and 5 minutes at about 105°C, as a post amplification incubation step to inactivate said polymerase; and
(iii) detecting the presence or absence of said biotinylated amplified target nucleic acids by reacting said biotinylated amplified target nucleic acids with an avidinenzyme conjugate, followed by reaction of said enzyme with a substrate reagent to produce a detectable colorimetric or chemiluminescent signal.

13. The method of claim 12, wherein said biotinylated amplified target nucleic acids are detected by contacting them

with an avidin-peroxidase conjugate, followed by reaction of peroxidase, in the presence of an oxidant, with either: luminol to produce a detectable chemiluminescent signal, or a leuco dye to produce a detectable colorimetric signal.